TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371		Attorney's Docket Number 045636-5052 U.S. Application No. Unassigned
International Application. No.	International Filing Date	Priority Date Claimed
PCT/FR00/01975	July 7, 2000	July 8, 1999

Title of Invention: ANTIMICROBIAL PEPTIDES DERIVED FROM MOLLUSKS

Applicants For EO/EO/US: Philippe ROCH, Guillaume MITTA, Florence HUBERT and Thierry NOEL

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)).
9. ☐ An oath or declaration of the inventors (35 U.S.C. § 371(c)(4)).
10. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. § 1.97 and § 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. § 3.28 and § 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☒ Other items or information:
    a. WO 01/04294
    b. PCT/IB/304
    c. PCT/IB/308
    d. Statement Accompanying Sequence Listing
    e. Diskette containing Sequence Listing CRF
    f. Paper Copy of Sequence Listing

10/030231 10/030231
JC13 Rec'd PCT/PTO 08 JAN 2002

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NUMBER
Unassigned	PCT/FR00/01975	045636-5052

15. ☒ The following fees are submitted:

Basic National Fee (37 C.F.R. § 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO.....$890.00
International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482).....$710.00
No international preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) but international search fee paid to USPTO (37 C.F.R. § 1.445(a)(2)).....$740.00
Neither international preliminary examination fee (37 C.F.R. § 1.482) nor international search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO.....$1,040.00
International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = $890.00

Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(e)). $

Claims	Number Filed	Number Extra	Rate	
Total Claims	- 20 =		X $18.00	$
Independent Claims	- 3 =		X $84.00	$
Multiple dependent claim(s) (if applicable)			+ $280.00	$
		TOTAL OF ABOVE CALCULATIONS		$
		Reduction by ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 C.F.R. §§ 1.9, 1.27, 1.28)		-$
		SUBTOTAL =		$
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(f)).				+$
		TOTAL NATIONAL FEE =		$890.00
Fee for recording the enclosed assignment (37 C.F.R. § 1.21(h)). The Assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3.31). $40.00 per property				$
		TOTAL FEES ENCLOSED =		$
		Amount to be refunded		$
		Amount to be charged		$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.
b. ☒ Please charge my Deposit Account No. 50-0310 in the amount of **$890.00**
c. ☒ **Except** for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. § 1.16 and § 1.17 which may be required, or credit any overpayment to Deposit Account No. 50-0310.

Customer No. 09629
SEND ALL CORRESPONDENCE TO:
Morgan, Lewis & Bockius LLP
1800 M Street, N.W.
Washington, D.C. 20036
Telephone: (202) 467-7000
Facsimile: (202) 467-7176

Elizabeth C. Weimar
Reg. No. 44,478

Submitted: January 8, 2002

PATENT
ATTORNEY DOCKET NO. 045636-5052-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Philippe ROCH *et al.***	)	
	)	
Application No.:	)	Group Art Unit: Unassigned
(Based on PCT/FR00/01975)	)	
Filed: January 8, 2002	)	Examiner: Unassigned
	)	
For: **ANTIMICROBIAL PEPTIDES DERIVED FROM MOLLUSKS**	)	

BOX SEQUENCE
Commissioner for Patents
Washington, D.C. 20231

STATEMENT ACCOMPANYING SEQUENCE LISTING

Dear Sir:

The undersigned hereby states upon information and belief that the Sequence Listing submitted concurrently herewith does not include matter which goes beyond the content of the application as filed and that the information recorded on the diskette submitted concurrently herewith is identical to the written Sequence Listing submitted herewith.

Respectfully submitted,

MORGAN, LEWIS & BOCKIUS LLP

Dated: January 8, 2002

By: Rachel B. Kapust
Rachel B. Kapust

Customer No. 09629
MORGAN, LEWIS & BOCKIUS LLP
1800 M Street, NW
Washington, D.C. 20036
Tel: 202-467-7000; Fax: 202-467-7258

PATENT
ATTORNEY DOCKET NO. 045636-5052

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of : Philippe **ROCH** et al.	Group Art Unit: Unassigned
U.S. National Phase Application Filed : January 8, 2002	Examiner: Unassigned
U.S. Application No.: To Be Assigned	
Date of National Stage Entry : Concurrently	
Based on PCT/FR00/01975 Filed : July 7, 2000	
For: **ANTIMICROBIAL PEPTIDES DERIVED FROM MOLLUSKS**	

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Please note that the English language title of the PCT publication has been changed to correct the spelling of MOLLUSKS.

Please substitute the following for the paragraph bridging pages one and two.

-- According to a preferred embodiment of an antimicrobial peptide in accordance with the invention, it comprises the following sequence (I) (1-letter code):

$HX_1HX_2CTSYX_3CX_4KFCGTAX_5CTX_6YX_7CRX_8LHX_9GKX_{10}CX_{11}CX_{12}HCSR$ (I)

in which: X_1 = P or S, X_2 = V or A, X_3 = Y or W, X_4 = S or G, X_5 = S or G, X_6 = R or H, X_7 = G or L, X_8 = N or V, X_9 = R or P, X_{10} = L or M, X_{11} = F or A, and X_{12} = L or V. (SEQ ID NO: 5)--

Please substitute the following for the paragraph on page 2, lines 6-11.

Advantageously, a peptide in accordance with the invention comprises one of the following sequences (Ia) or (Ib) (1-letter code):

HSHACTSYWCGKFCGTASCTHYLCRVLHPGKMCACVHCSR (Ia) (SEQ ID NO:6)

HPHVCTSYYCSKFCGTAGCTRYGCRNLHRGKLCFCLHCSR (Ib) (SEQ ID NO:7).

Please substitute the following amended claims for claims 2 – 4.

2. (Amended) The peptide of claim 1, comprising the following sequence (I):

$HX_1HX_2CTSYX_3CX_4KFCGTAX_5CTX_6YX_7CRX_8LHX_9GKX_{10}CX_{11}CX_{12}HCSR$ (I)

in which: X_1 = P or S, X_2 = V or A, X_3 = Y or W, X_4 = S or G, X_5 = S or G, X_6 = R or H, X_7 = G or L, X_8 = N or V, X_9 = R or P, X_{10} = L or M, X_{11} = F or A, and X_{12} = L or V (SEQ ID NO: 5).

3. (Amended) The peptide of claim 2, chosen from the group consisting of:

- a peptide comprising the following sequence (Ia):

HSHACTSYWCGKFCGTASCTHYLCRVLHPGKMCACVHCSR (Ia) (SEQ ID NO: 6)

- a peptide comprising the following sequence (Ib):

HPHVCTSYYCSKFCGTAGCTRYGCRNLHRGKLCFCLHCSR (Ib) (SEQ ID NO: 7).

4. (Amended) A nucleic acid comprising a sequence encoding the peptide as claimed in claim 1.

Please cancel claims 5, 9 and 10.

Please add the following new claims 11 – 18.

11. A nucleic acid comprising a sequence which encodes a peptide as claimed in claim 2.

12. A nucleic acid comprising a sequence which encodes a peptide as claimed in claim 3.

13. A method of detecting a nucleic acid as claimed in claim 4 comprising screening a nucleic acid library with a fragment of more than 15 base pairs of the coding region of either SEQ ID NO:1 or SEQ ID NO: 3.

14. A prokaryotic or eukaryotic cell transformed with a nucleic acid sequence as claimed in claim 11.

15. A method of producing a myticin antimicrobial peptide which has a molecular mass of approximately 4.5 kDa, a pI of approximately 8.7 and containing 8 cysteine residues, comprising culturing the transformed cell of claim 8 under conditions effective for the expression of the nucleic acid which encodes the myticin peptide.

16. A method of producing a myticin antimicrobial peptide which has a molecular mass of approximately 4.5 kDa, a pI of approximately 8.7 and containing 8 cysteine residues, comprising culturing the transformed cell of claim 14 under conditions effective for the expression of the nucleic acid which encodes the myticin peptide.

17. A method of treating a bacterial, fungal or parasitic infection in a patient or animal comprising administration of an amount of the myticin antimicrobial peptide of claim 1 effective to inhibit further growth of the infectious organism.

18. A method of treating a bacterial, fungal or parasitic infection in a patient or animal comprising administration of an amount of the myticin antimicrobial peptide of claim 2 effective to inhibit further growth of the infectious organism.

REMARKS

Applicants respectfully submit that no prohibited new matter has been introduced by this Preliminary Amendment and that claims 1- 4 and 6-18 including amended claims 2 to 4 and newly added claims 11-18 are drawn to the same invention as claims 1-10 of International Application PCT/FR00/01975. The changes to the claims were made to bring the claims into compliance with US rules and do not represent a narrowing of the claimed subject matter. The changes designed to avoid objections are multiply dependent claims have been removed to avoid multiply dependent claims depending from multiply dependent claims (see original claims 4, 9 and 10); the re-phrasing of original claim 10 which was drafted as a "use" claim has been correctly presented as method claims 17 and 18; sequence identifiers have been introduced where necessary; the sequence options for amino acid X_{12} in the sequence of claim 2 have been corrected to reflect the options presented with respect to the two sequences in original claim 3. Similarly, the sequence set forth in claim 2 has been corrected in the paragraph bridging pages 1 and 2 of the specification to reflect the sequence options for amino acid X_{12} as lysine or valine as indicated in the sequences set forth in original claim 3. The method of treating infections claimed in new claims 17 and 18 is further supported by Example 2 on pages 8 - 11 of the specification.

A marked-up version of the changes to the specification and comparing amended claims 2-4 to original claims 2-4 is attached.

If there are any other fees due in connection with the filing of this Preliminary Amendment, please charge the fees to our Deposit Account No. 50-0310.

Respectfully submitted,
Morgan, Lewis & Bockius LLP

Elizabeth C. Weimar

Elizabeth C. Weimar
Registration No. 44,478

Date: **January 8, 2002**
Morgan, Lewis & Bockius LLP
Customer No. **009629**
1800 M Street, N.W.
Washington, D.C. 20036-5869

Marked-up Version of the Amendments

As to the substitution of the paragraph bridging pages one and two:

According to a preferred embodiment of an antimicrobial peptide in accordance with the invention, it comprises the following sequence (I) (1-letter code):

$HX_1HX_2CTSYX_3CX_4KFCGTAX_5CTX_6YX_7CRX_8LHX_9GKX_{10}CX_{11}CX_{12}HCSR$ (I)

in which: X_1 = P or S, X_2 = V or A, X_3 = Y or W, X_4 = S or G, X_5 = S or G, X_6 = R or H, X_7 = G or L, X_8 = N or V, X_9 = R or P, X_{10} = L or M, X_{11} = F or A, and X_{12} = L or [H] V (SEQ ID NO: 5).--

As to the substitution of the paragraph on lines 6-11 of page two:

Advantageously, a peptide in accordance with the invention comprises one of the following sequences (Ia) or (Ib) (1-letter code):

HSHACTSYWCGKFCGTASCTHYLCRVLHPGKMCACVHCSR (Ia) (SEQ ID NO:6)

HPHVCTSYYCSKFCGTAGCTRYGCRNLHRGKLCFCLHCSR (Ib) (SEQ ID NO:7).

As to amended claims 2-4:

2. (Amended) The peptide of claim 1, comprising the following sequence (I):

$HX_1HX_2CTSYX_3CX_4KFCGTAX_5CTX_6YX_7CRX_8LHX_9GKX_{10}CX_{11}CX_{12}HCSR$ (I)

in which: X_1 = P or S, X_2 = V or A, X_3 = Y or W, X_4 = S or G, X_5 = S or G, X_6 = R or H, X_7 = G or L, X_8 = N or V, X_9 = R or P, X_{10} = L or M, X_{11} = F or A, and X_{12} = L or [H] V (SEQ ID NO: 5).

3. (Amended) The peptide of claim 2, chosen from the group consisting of:

- a peptide comprising the following sequence (Ia):

HSHACTSYWCGKFCGTASCTHYLCRVLHPGKMCACVHCSR (Ia) (SEQ ID NO: 6)

- a peptide comprising the following sequence (Ib):

HPHVCTSYYCSKFCGTAGCTRYGCRNLHRGKLCFCLHCSR (Ib) (SEQ ID NO: 7).

4. (Amended) A nucleic acid comprising a sequence encoding the peptide as claimed in [any one of] claim[s] 1 [to 3].

ANTIMICROBIAL PEPTIDES ON MOLLUSKS

The invention relates to novel antimicrobial peptides produced by mollusks.

Polypeptides possessing antimicrobial properties are produced by a large variety of species (animal or plant species), in which they contribute to nonspecific mechanisms of defense against infections.

In the case of bivalve mollusks, to date, in *Mytilus galloprovincialis*, a peptide named MGD-1 has been identified, which is related to insect defensins [HUBERT et al., Eur. J. Biochem., 240, 302-306, (1996)]; peptides of the defensin family have also been demonstrated in *Mytilus edulis*, as have peptides named "mytilins" [CHARLET et al., J. Biol. Chem., 271, 21808-21813, (1996)].

The inventors have now demonstrated novel antimicrobial peptides produced by *Mytilus galloprovincialis*, which are different from the MGD1 defensins and form the previously known mytilins.

A subject of the present invention is antimicrobial peptides, hereinafter named: "myticins", which have the following characteristics:

- their molecular mass is approximately 4.5 kDa;
- their pI is approximately 8.7;
- they comprise 8 cysteine residues.

According to a preferred embodiment of an antimicrobial peptide in accordance with the invention, it comprises the following sequence (I) (1-letter code):

$$HX_1HX_2CTSYX_3CX_4KFCGTAX_5CTX_6YX_7CRX_8LHX_9GKX_{10}CX_{11}CX_{12}HCSR \quad (I)$$

in which: X_1 = P or S, X_2 = V or A, X_3 = Y or W, X_4 = S or G, X_5 = S or G, X_6 = R or H, X_7 = G or L, X_8 = N or V, X_9 = R or P, X_{10} = L or M, X_{11} = F or A, and X_{12} = L or V.

in which: X_1 = P or S, X_2 = V or A, X_3 = Y or W, X_4 = S or G, X_5 = S or G, X_6 = R or H, X_7 = G or L, X_8 = N or V, X_9 = R or P, X_{10} = L or M, X_{11} = F or A, and X_{12} = L or H.

Advantageously, a peptide in accordance with the invention comprises one of the following sequences (Ia) or (Ib) (1-letter code):

HSHACTSYWCGKFCGTASCTHYLCRVLHPGKMCACVHCSR (Ia)
HPHVCTSYYCSKFCGTAGCTRYGCRNLHRGKLCFCLHCSR (Ib)

The sequences (Ia) and (Ib) represent the mature forms, isolated from the hemolymph of *Mytilus galloprovincialis*, of 2 myticins named Myticin a and Myticin b, the cDNAs of which have also been cloned by the inventors. By way of illustration of the subject of the present invention, the characteristics of Myticin a and Myticin b are more specifically indicated below.

The cDNA sequence and the polypeptide sequence of Myticin a are represented in the attached sequence listing, under the numbers SEQ ID NO: 1 and SEQ ID NO: 2. The cDNA sequence and the polypeptide sequence of Myticin b are represented in the attached sequence listing, under the numbers SEQ ID NO: 3 and SEQ ID NO: 4.

The 40 amino acid active peptide, corresponding to the sequence (I), and more particularly to one of the sequences (Ia) and (Ib), is flanked by a 20 amino acid signal sequence and by a 36 amino acid C-terminal peptide. The signal sequence is thought to enable the addressing of the translation product toward the endoplasmic reticulum. The C-terminal peptide would then enable addressing toward the cytoplasmic granules in which the myticins are stored in mature form, and/or protection of the cell against possible cytolytic activity of the mature peptide.

The molecular mass of the mature form of Myticin a is 4438 Da; the molecular mass of the mature form of Myticin b is 4562 Da.

Myticins exhibit no significant homology with the known antimicrobial peptides in the prior art, and define a novel group of antimicrobial peptides.

Myticins may be obtained by extraction from the mollusks which produce them, by peptide synthesis or, advantageously, by genetic engineering, expressing at least one nucleic acid sequence encoding a myticin, in a suitable host cell.

The present invention also encompasses nucleic acids comprising a sequence encoding a myticin, as defined above.

Nucleic acids in accordance with the invention may be obtained by screening nucleic acid libraries using oligonucleotides derived from the sequences SEQ ID NO: 1 or SEQ ID NO: 3, or from the sequences complementary thereto. The oligonucleotides which can be used for this purpose are also part of the subject of the present invention; advantageously, these oligonucleotides comprise at least 15 bp, and preferably at least 20 bp, of the coding region of one of the sequences SEQ ID NO: 1 or SEQ ID NO: 3, or of the sequence complementary thereto.

The nucleic acids in accordance with the invention also encompass the expression cassettes comprising at least one nucleic acid sequence encoding a myticin, placed under the transcriptional control of a suitable promoter.

The term "suitable promoter" is intended to mean any promoter which is functional in the host cell intended

to harbor the expression cassette. It may be a constitutive promoter or an inducible promoter; it may also be, when the cassette is intended for the expression of a myticin in an animal or a plant, a tissue-specific promoter.

An expression cassette in accordance with the invention may also comprise at least one sequence encoding a suitable addressing sequence; said addressing sequence may be chosen from those which are naturally associated with myticins, such as the signal sequences and/or the C-terminal sequences associated with the Myticin a and Myticin b isoforms described above; it is also possible to choose one or more heterologous addressing sequences which are functional in a given host cell: they may in particular be sequences which allow the addressing of a myticin toward a given cellular compartment, or its secretion into the culture medium.

A subject of the invention is also:

- recombinant vectors, characterized in that they comprise at least one nucleic acid sequence in accordance with the invention, encoding a myticin, and, in particular, vectors comprising an expression cassette as defined above.

- prokaryotic or eukaryotic cells transformed with at least one nucleic acid sequence in accordance with the invention. They may be cells in culture or cells which form part of an animal or plant multicellular organism. The nucleic acid sequence in accordance with the invention present in a transformed cell may be either incorporated into the chromosomal DNA of said cell, or be carried by an extrachromosomal vector.

A subject of the invention is also a method for producing a myticin, characterized in that it comprises

expressing said myticin in at least one transformed cell in accordance with the invention.

The myticins in accordance with the invention may be expressed in cultures of cells transformed using techniques similar to those used for antimicrobial peptides of the prior art, for example in insect cells, as described by HELLERS et al. [Eur. J. Biochem. 199, pp. 435-439, (1991)] for cecropins, or in yeast, as described by REICHHART et al. [Invertebrate Reproduction and Development, 21, pp. 15-24, (1992)].

They may also be expressed in transgenic animals or plants, in order to increase the resistance thereof to infections, as described, for example, by JAYNES et al. [Plant Science, 89, pp. 43-53 (1993)] in the case of peptides analogous to cecropin B, expressed in transgenic tobacco plants, or by NORELLI et al. [Euphytica, 77, pp. 123-128 (1994)] for transgenic apple tree plants expressing the attacin-E gene.

The myticins can be used in particular for producing anti-infectious, for example antibacterial or fungicidal, products, and in particular medicinal products.

Such products are applied for preventing and treating various microbial diseases, in very varied sectors, in particular in the domains of health and of agriculture, and in that of aquaculture, in order to limit the development of infectious diseases in breeding stocks.

The present invention will be more clearly understood from the further description which follows, which refers to examples of purification and of characterization of the myticins.

EXAMPLE 1: ISOLATION OF ANTIMICROBIAL PEPTIDES FROM THE HEMOLYMPH OF *MYTILUS GALLOPROVINCIALIS*

Preparation of the hemolymph fractions

An immune reaction is induced in adult mussels (*Mytilus galloprovincialis*) according to the following protocol: the liquid is removed from the shell, and 100 µl of a suspension of bacteria (10^9 bacteria/ml) or of fungi (suspension of hyphae at 1 OD at 600 nm), heat-killed beforehand, are injected into the adductor muscle. The hemolymph (approximately 0.5 ml/animal) is removed from the posterior adductor muscle using a syringe, in the presence of one volume of MAS (Modified Alsevier Solution) anti-aggregating buffer, and immediately centrifuged at 800g for 15 min at 4°C. Aprotinin (5 µg/ml) is added to the supernatant, corresponding to the plasmatic fraction, which is frozen (-80°C) until use, and the cell pellet is dried and stored at -80°C until use.

Purification of myticins

Plasmatic fraction: The plasma is diluted (1:1 v/v) in water sterilized by ultrafiltration (MilliQ), to which 0.1% of trifluoroacetic acid has been added. The pH is brought to 3.9 by adding 1 M HCl, with stirring, in an iced water bath for 30 min. After centrifugation (10 000 g, 20 min, 4°C), the supernatant is harvested and kept at 4°C until use.

Hemocytes: After thawing, the hemocyte pellet is resuspended in 5 volumes of 50 mM Tris buffer, pH 8.7, containing 50 mM NaCl, and homogenized. After centrifugation (10 000 g, 20 min, 4°C), the pellet containing the cellular organelles is taken up in 3 volumes of 2 M acetic acid and treated by sonication (3 × 30 s) in an iced water bath. After removal of the debris by

centrifugation (10 000 g, 20 min, 4°C), the acid extract is stored at 4°C until use.

HPLC purification

The plasmatic fraction or the acid extracts of hemocytes are loaded onto SEP-PAK C18 VAC columns (WATERS ASSOCIATES) pre-equilibrated with acidified (0.05% trifluoroacetic acid) water. After washing with the acidified water, 2 successive elutions are carried out with solutions of acetonitrile at 10% and 40% in acidified (0.05% trifluoroacetic acid) water. The fractions obtained are lyophilized and reconstituted with ultrafiltered water, before being subjected to reverse-phase HPLC chromatography.

All the HPLC purification steps were carried out on a BECKMAN GOLD HPLC system equipped with a BECKMAN 168 detector. The elution is monitored by measuring UV absorption at 225 nm.

Step 1: The fractions eluted on SEP-PAK at 40% of acetonitrile are loaded onto a SEPHASIL C18 reverse-phase HPLC column (250 mm × 4.1 mm) (PHARMACIA). Elution is carried out with a linear gradient of 5 to 50% of acetonitrile in the acidified water, for 90 min at a flow rate of 0.9 ml/min. The fractions corresponding to the absorbence peaks are collected in polypropylene tubes (MICROSORB, 75 × 12 mm, NUNC IMMUNOTUBES), dried under vacuum and reconstituted with ultrafiltered water, prior to testing their antimicrobial activity.

Step 2: The active fractions recovered at the end of step 1 are loaded onto a SEPHASIL C8 reverse-phase HPLC column (250 mm × 4.1 mm) (PHARMACIA). The elution is carried out, at a flow rate of 0.9 ml/min, with a linear gradient of 20 to 30% of acetonitrile in the acidified water for 40 min.

Step 3: The active fractions recovered at the end of step 2 are loaded onto a SEPHASIL C18 column (250 mm × 4.1 mm) (PHARMACIA), using the biphasic gradient described in step 2, at a flow rate of 0.9 ml/min.

Step 4: The final purification step is carried out on a DELTA PAK HPI C18 reverse-phase column (2 × 150 mm) (WATERS ASSOCIATES), using the biphasic gradient described in step 2, at a flow rate of 0.3 ml/min.

EXAMPLE 2: ANTIMICROBIAL ACTIVITY OF THE PEPTIDES OBTAINED

Microorganisms used:

The list of the microorganisms used to determine the antimicrobial activities of Myticin a and of Myticin b is indicated below, in table 1.

Antibacterial assays and determination of the MBC:

The minimum bactericidal concentration (MBC) of the peptides was determined according to the protocol described by HANCOCK et al. [http://www.interchg.ubc.ca/bobh/methods.htm].

A series of successive doubling dilutions of the peptides, in an aqueous solution containing 0.01% of acetic acid and 0.2% of bovine serum albumin (BSA), is prepared.

10 µl aliquots of each dilution are incubated in sterile 96-well polypropylene microtitration plates, in the presence of 100 µl of bacterial suspension at a starting optical density of A_{600} = 0.001, in MUELLER HINTON BROTH liquid medium. The incubation is carried out for 18 h at 37°C with stirring, except in the case of the marine bacteria, for which the incubation is carried out 25°C. The MBC is determined by plating out,

onto solid MUELLER HINTON AGAR medium, the content of the wells corresponding to the first 3 dilutions for which no bacterial growth is observed, and incubating at 37°C for 18 hours. The lowest concentration of peptide which prevents any residual formation of colonies corresponds to the MBC.

Antifungal activity:

The antifungal activity was determined by calculating the MIC (minimum inhibitory concentration) in a test of inhibition of *Fusarium oxysporum* growth in liquid phase, according to the protocol described by FELHBAUM et al. [J. Biol. Chem., 269: 33159-63, (1994)].

A series of successive doubling dilutions of the peptides is prepared as indicated above for determining the antibacterial activity.

80 µl of spores suspended (final concentration 10^4 spores/ml) in Potato Dextrose Broth medium (DIFCO) are added to 10 µl of peptide solution in sterile 96-well polypropylene microtitration plates. The final volume is adjusted to 100 µl by adding water. The growth inhibition is determined after incubation for 24 hours at 25°C in the dark, by observation under a microscope and measurement of the increase in the OD_{600}. The value of the MIC corresponds to a range (a-b) of peptide concentrations, in which (a) represents the highest concentration at which growth is observed, and (b) represents the lowest concentration which induces 100% growth inhibition.

Antiprotozoan activity:

The oyster-parasite protozoan *Perkinsus marinus* is cultured in DMEM medium (GIBCO), according to the protocol described by GAUTHIER and VASTA [J. Invertebr. Pathol., 66, 156-168, (1995)].

10 µM of purified peptide are added to 4×10^4 *P. marinus*, in seawater (final volume 20 µl). The mixture is incubated for 1 hour at room temperature. The viability of the parasites is estimated by staining with acridine orange and with ethidium bromide, as described by MORVAN et al. [J. Invertebr. Pathol., 69, 177-82 (1997)]. The maximum viability is evaluated, as a positive control, in samples to which the peptide has not been added.

The results of the various experiments carried out, for the Myticin a and Myticin b peptides, are illustrated by table 1 below; the biological activities are expressed in µM.

TABLE 1

	Myticin a	Myticin b
BACTERIA		
Gram-positive		
Micrococcus luteus	2.25-4.5	1-2
Bacillus megaterium	2.25-4.5	1-2
Staphylococcus aureus	>20	>20
Listeria monocytogenes	>20	>20
Aerococcus viridans	4.5-9	2-4
Enterococcus faecalis	>20	N.D.
Gram-negative		
Escherichia coli D31	>20	10-20
Salmonella newport	>20	>20
S. typhimurium	>20	>20
Brucella suis	>20	>20
Pseudomonas aeruginosa	>20	N.D.
Enteromonas aerogenes	>20	N.D.
Vibrio alginolyticus	>20	>20
V. vulnificus	>20	>20
V. splendidus	>20	>20

	Myticin a	Myticin b
FUNGI		
Fusarium oxysporum	>20	5-10
OYSTER-PARASITE PROTOZOAN		
Perkinsus marinus	>20	>20

N.D.: not determined

These results show that the 2 peptides are active, in particular on *Micrococcus luteus;* the Myticin b peptide also appears to be more active than the Myticin a peptide on *Micrococcus luteus, Escherichia coli* and *Fusarium oxysporum.*

EXAMPLE 3: MYTICIN PEPTIDE cDNA CLONING

A cDNA library was constructed in the ZAP EXPRESS vector (STRATAGENE) using total poly(A)$^+$ RNAs from adult mussel hemocytes. A DNA probe representing 83 bp of the Myticin a cDNA was constructed using the PCR SCRIPT Amp (SK+) cloning kit (STRATAGENE), and labeled by random priming using the READY-TO-GO DNA labeling kit (PHARMACIA), and used to screen the DNA library transferred onto HYBOND-N membranes (AMERSHAM). Hybridizations at high stringency were carried out overnight at 65°C in 5X Denhardt's solution, 5X SSPE, 0.1% SDS, 100 µg/ml of salmon sperm DNA. The filters, rinsed beforehand at 65°C in 0.5 X SSC solution containing 0.1% SDS, were autoradiographed. A secondary screening was carried out in order to purify the positive clones. The phagemids were obtained by *in vivo* excision and both of their strands were sequenced.

110 positive clones were obtained. Among these clones, 4 were sequenced, and correspond to the Myticin a and Myticin b peptides.

In both cases, the amino acid sequence deduced from the open reading frame begins with a 20 amino acid signal

peptide; this signal peptide is directly followed, at its C-terminal end, by a 40 amino acid peptide beginning with a histidine residue, which corresponds to the active form of the peptide; this active peptide is followed by a 36 amino acid C-terminal extension.

CLAIMS

1. An antimicrobial peptide, named myticin, characterized in that it can be obtained from a bivalve mollusk, and in that

- its molecular mass is approximately 4.5 kDa;
- its pI is approximately 8.7;
- it comprises 8 cysteine residues.

2. The peptide as claimed in claim 1, characterized in that it comprises the following sequence (I):

$HX_1HX_2\underline{C}TSYX_3\underline{C}X_4KF\underline{C}GTAX_5\underline{C}TX_6YX_7\underline{C}RX_8LHX_9GKX_{10}\underline{C}X_{11}\underline{C}X_{12}H\underline{C}SR$ (I)

in which: X_1 = P or S, X_2 = V or A, X_3 = Y or W, X_4 = S or G, X_5 = S or G, X_6 = R or H, X_7 = G or L, X_8 = N or V, X_9 = R or P, X_{10} = L or M, X_{11} = F or A, and X_{12} = L or V.

3. The peptide as claimed in claim 2, chosen from the group consisting of:

- a peptide comprising the following sequence (Ia):

HSHACTSYWCGKFCGTASCTHYLCRVLHPGKMCACVHCSR (Ia)

- a peptide comprising the following sequence (Ib):

HPHVCTSYYCSKFCGTAGCTRYGCRNLHRGKLCFCLHCSR (Ib).

4. A nucleic acid comprising a sequence encoding the peptide as claimed in any one of claims 1 to 3.

5. A method for producing the nucleic acid as claimed in claim 4, characterized in that it comprises screening a nucleic acid library using a fragment of more than

15 bp of the coding region of a sequence chosen from SEQ ID NO: 1 and SEQ ID NO: 3.

6. An expression cassette comprising at least one nucleic acid sequence as claimed in claim 4, under the transcriptional control of a suitable promoter.

7. A recombinant vector, characterized in that it comprises at least one nucleic acid sequence as claimed in claim 4.

8. A prokaryotic or eukaryotic cell transformed with a nucleic acid sequence as claimed in claim 4.

9. A method for producing the peptide as claimed in any one of claims 1 to 3, characterized in that it comprises expressing a nucleic acid as claimed in claim 4, in at least one transformed cell as claimed in claim 8.

10. The use of the peptide as claimed in any one of claims 1 to 3, for producing an antimicrobial agent.

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(71) Déposants *(pour tous les États désignés sauf US)*: **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)** [FR/FR]; 3, rue Michel-Ange, F-75794 Paris Cedex 16 (FR). **INSTITUT FRANCAIS DE RECHERCHE POUR L'EXPLOITATIONDE LA MER (IFREMER)** [FR/FR]; 155, rue Jean-Jacques Rousseau, F-92138 Issy-les-Moulineaux Cedex (FR).

(72) Inventeurs; et

(75) Inventeurs/Déposants *(pour US seulement)*: **ROCH, Philippe** [FR/FR]; 113, rue du Fesquet A1, F-34080 Montpellier (FR). **MITTA, Guillaume** [FR/FR]; Résidence Le Maurétania N° 10, Impasse Georges Costes, F-34000 Montpellier (FR). **HUBERT, Florence** [FR/FR]; Résidence Le Gauguin, B3-333, 75, Avenue du Pont Trinquat, F-34000 Montpellier (FR). **NOEL, Thierry** [FR/FR]; 8, rue Yves Montand, F-34830 Clapiers (FR).

(74) Mandataires: **VIALLE-PRESLES, Marie-José** etc.; Cabinet Ores, 6, avenue de Messine, F-75008 Paris (FR).

(81) États désignés *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) États désignés *(régional)*: brevet ARIPO (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), brevet eurasien (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), brevet européen (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), brevet OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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— *Avec rapport de recherche internationale.*

En ce qui concerne les codes à deux lettres et autres abréviations, se référer aux "Notes explicatives relatives aux codes et abréviations" figurant au début de chaque numéro ordinaire de la Gazette du PCT.

WO 01/04294 A1

(54) **Title:** ANTIMICROBIAL PEPTIDES DERIVED FROM MOLLUSCS

(54) **Titre:** PEPTIDES ANTI-MICROBIENS DE MOLLUSQUES

(57) **Abstract:** The invention concerns an antimicrobial peptide, called myticin, characterised in that it can be obtained from a bivalve mollusc shellfish, and its molecular mass is about 4.5 kDa; its pI is about 8.7; it comprises 8 cystein radicals. The invention also concerns its preparation and its uses. The invention further concerns a nucleic acid coding for said peptide.

(57) **Abrégé:** Peptide anti-microbien, dénommé myticine, caractérisé en ce qu'il est susceptible d'être obtenu à partir d'un mollusque bivalve, et en ce que sa masse moléculaire est d'environ 4,5 kDa; son pI est d'environ 8,7; il comprend 8 résidus cystéine, son procédé de préparation et ses applications. Acide nucléique codant pour ledit peptide.

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

ATTORNEY DOCKET NO.: 045636-5052

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ANTIMICROBIAL PEPTIDES DERIVED FROM MOLLUSCS

the specification of which:

is attached hereto; or

was filed as United States application Serial No. _____ on _____ and was amended on _____ (if applicable); or

was filed as PCT international application Number PCT/FR00/01975 on July 7, 2000 and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office information which is material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN APPLICATION(S):

COUNTRY (if PCT, indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
France	99/08858	8 July 1999	[X] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No

Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO.: 045636-5052

I hereby claim the benefits under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

U.S. PROVISIONAL APPLICATIONS

U.S. PROVISIONAL APPLICATION NO.	U S. FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or §365(c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT:

U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NO.	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number.

Customer Number: 009629

Direct Telephone Calls To:
(name and telephone number)

Elizabeth C. Weimar
202-467-7000

Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO. 045636-5052

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF SOLE OR FIRST INVENTOR 1-00	Philippe ROCH	
RESIDENCE & CITIZENSHIP	Montpellier, France FRX	COUNTRY OF CITIZENSHIP France
POST OFFICE ADDRESS	113, rue du Fesquet A1, F-34080 Montpellier, France	
FIRST OR SOLE INVENTOR'S SIGNATURE		DATE 28/fév 2002
FULL NAME OF SECOND INVENTOR 2-00	Guillaume MITTA	
RESIDENCE & CITIZENSHIP	Montpellier, France FRX	COUNTRY OF CITIZENSHIP France
POST OFFICE ADDRESS	Résidence Le Maurétania N° 10, Impasse Georges Costes, F-34000 Montpellier, France	
SECOND INVENTOR'S SIGNATURE		DATE 28 février 2002
FULL NAME OF THIRD INVENTOR 3-00	Florence HUBERT	
RESIDENCE & CITIZENSHIP	Montpellier, France FRX	COUNTRY OF CITIZENSHIP France
POST OFFICE ADDRESS	Résidence Le Gauguin, B3-333, 75, Avenue du Pont Trinquat, F-34000 Montpellier, France	
THIRD INVENTOR'S SIGNATURE		DATE 28/02/02

Listing of Inventors Continued on attached page(s) [X] Yes [ ] No

Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO.: 045636-5052

FULL NAME OF FOURTH INVENTOR	Thierry NOEL	
RESIDENCE & CITIZENSHIP	Clapiers, France FRX	COUNTRY OF CITIZENSHIP France
POST OFFICE ADDRESS	8, rue Yves Montand, F-34830 Clapiers, France	
FOURTH INVENTOR'S SIGNATURE		DATE 28/02/2002
FULL NAME OF FIFTH INVENTOR		
RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		
FIFTH INVENTOR'S SIGNATURE		DATE
FULL NAME OF SIXTH INVENTOR		
RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		
SIXTH INVENTOR'S SIGNATURE		DATE
FULL NAME OF SEVENTH INVENTOR		
RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		
SEVENTH INVENTOR'S SIGNATURE		DATE

Listing of Inventors Continued on attached page(s) [ ] Yes [X] No

SEQUENCE LISTING

<110> ROCH, Philippe
MITTA, Guillaume
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aag ttc ccg ttt gga gca act caa gat gct aaa agt atg aac gaa ctg 243
Lys Phe Pro Phe Gly Ala Thr Gln Asp Ala Lys Ser Met Asn Glu Leu
45 50 55

gaa tac act cca ata atg aag tcg atg gaa aat ttg gac aac gga atg 291
Glu Tyr Thr Pro Ile Met Lys Ser Met Glu Asn Leu Asp Asn Gly Met
60 65 70

gat atg tta taagcaaact tatgacatga agatcacaac tgtatacttt 340
Asp Met Leu
75

tgctattcct gtatccgctt tactcctttc ttcacacttt gtacggaatc cgtcaacaga 400

aaattcatca tcaacttgaa aactaacaaa agatgtgtcg cacacgttac actcaccagt 460

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atctctgaca gaagaagtaa ctgttaacaa gaaatactgt tttccctcaa gttattaaaa 580

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Phe Cys Gly Thr Ala Gly Cys Thr Arg Tyr Gly Cys Arg Asn Leu His
35 40 45

Arg Gly Lys Leu Cys Phe Cys Leu His Cys Ser Arg Val Lys Phe Pro
50 55 60

Phe Gly Ala Thr Gln Asp Ala Lys Ser Met Asn Glu Leu Glu Tyr Thr
65 70 75 80

Pro Ile Met Lys Ser Met Glu Asn Leu Asp Asn Gly Met Asp Met Leu
85 90 95

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Arg Gly Lys Leu Cys Phe Cys Leu His Cys Ser Arg Val Lys Phe Pro
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Phe Gly Ala Thr Gln Asp Ala Lys Ser Met Asn Glu Leu Glu Tyr Thr
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			20					25					30		
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		35					40								

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His	Pro	His	Val	Cys	Thr	Ser	Tyr	Tyr	Cys	Ser	Lys	Phe	Cys	Gly	Thr
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Ala	Gly	Cys	Thr	Arg	Tyr	Gly	Cys	Arg	Asn	Leu	His	Arg	Gly	Lys	Leu
			20					25					30		
Cys	Phe	Cys	Leu	His	Cys	Ser	Arg								
		35					40								